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Generalized Lymphoproliferative Disease in Mice, Caused by a Point Mutation in the Fas Ligand

Tomohiro Takahashi,* Masato Tanaka,*
Camilynn I. Brannan,† Nancy A. Jenkins,†
Neal G. Copeland,† Takashi Suda,*
and Shigekazu Nagata*
*Osaka Bioscience Institute
6–2–4 Furuedai
Suita
Osaka 565
Japan
†Mammalian Genetics Laboratory
Advanced BioScience Laboratories–Basic
Research Program
National Cancer Institute–Frederick Cancer Research
and Development Center
Frederick, Maryland 21702

Summary

Mice homozygous for *lpr* (lymphoproliferation) or *gld* (generalized lymphoproliferative disease) develop lymphadenopathy and suffer from autoimmune disease. The *lpr* mice have a mutation in a cell-surface protein, Fas, that mediates apoptosis. Fas ligand (FasL) is a tumor necrosis factor (TNF)-related type II membrane protein and binds to Fas. Here, mouse *Fasl* gene was isolated and localized to the *gld* region of mouse chromosome 1. Activated splenocytes from *gld* mice express *Fasl* mRNA. However, FasL in *gld* mice carries a point mutation in the C-terminal region, which is highly conserved among members of the TNF family. The recombinant *gld* FasL expressed in COS cells could not induce apoptosis in cells expressing Fas. These results indicate that *lpr* and *gld* are mutations in *Fas* and *Fasl*, respectively, and suggest important roles of the Fas system in development of T cells as well as cytotoxic T lymphocyte–mediated cytotoxicity.

Introduction

While T lymphocytes respond to a variety of foreign antigens, they do not react to self-components. The education or repertoire selection of T cells occurs during their development. T cell progenitors arise in the bone marrow and then migrate into the thymus, where interactions of precursor T cells with thymic epithelial cells promote maturation of the cells (van Ewijk, 1991; von Boehmer, 1988). Immature T cells that recognize autoantigens are deleted by negative selection, a process that occurs by apoptosis (Murphy et al., 1990). T cells carrying the T cell receptor, which do not recognize self-MHC as a restriction element and cannot receive positive selection, are neglected and deleted by programmed cell death (Blackman et al., 1990; Ramsdell and Fowlkers, 1990). It has been estimated that >95% of immature T cells die in the thymus (Scollay et al., 1980; Egerton et al., 1990). In addition to deletion in the thymus, some mature $CD4^+$ or $CD8^+$ single-positive T cells reacting with self-antigens are deleted in the periphery (Jones et al., 1990; Kawabe and Ochi, 1991; Russell et al., 1991; Webb et al., 1990). This process is known as peripheral clonal elimination or extrathymic tolerance and may also occur by apoptosis (Kabelitz et al., 1993; Kawabe and Ochi, 1991).

Mice homozygous for *lpr* (lymphoproliferation) or *gld* (generalized lymphoproliferative disease) mutations accumulate a large amount of nonmalignant $CD4^-$ $CD8^-$ T cells in the spleen and lymph nodes (Andrews et al., 1978; Cohen and Eisenberg, 1991; Roths et al., 1984). These mice also suffer from autoimmune disease like systemic lupus erythematosus by producing autoantibodies, anti-DNA and rheumatoid factor, and die around 5 months of age. The *lpr* and *gld* mutations are nonallelic and are localized on mouse chromosomes 19 and 1, respectively (Roths et al., 1984; Watanabe et al., 1991). Although the *lpr* and *gld* mutations were originally thought of as mutations in the common metabolic pathway (Davidson et al., 1985), bone marrow transplantation experiments suggested that they were mutations in an interacting pair of molecules (Allen et al., 1990). In this model, the *lpr* product was suggested to be a receptor expressed in both bone marrow–derived cells and peripheral cells, while the *gld* product was considered to be a soluble cytokine or membrane-associated protein expressed in bone marrow–derived cells.

Fas antigen (Fas) is a 45 kd protein belonging to the TNF (tumor necrosis factor)/NGF (nerve growth factor) receptor family and mediates apoptosis (Itoh et al., 1991; Nagata, 1994). Fas is expressed in the thymus, liver, heart, and ovary (Watanabe-Fukunaga et al., 1992b). Genetic analysis localized the *Fas* gene near the *lpr* locus on mouse chromosome 19, and characterization of its gene structure indicated that *lpr* is a mutation of the *Fas* gene (Watanabe-Fukunaga et al., 1992a). Two alleles, *lpr* and lpr^{cg}, have been identified (Cohen and Eisenberg, 1991). In *lpr*, an early transposable element is inserted into intron 2 of the *Fas* gene, which causes premature termination and aberrant splicing of the *Fas* transcript (Adachi et al., 1993). In lpr^{cg}, a point mutation in the *Fas* gene was identified that causes a replacement of isoleucine with asparagine and abolishes the ability of Fas to transduce the apoptotic signal (Watanabe-Fukunaga et al., 1992a).

The structure of Fas suggested that Fas is a receptor for an unknown cytokine. Recently, we purified the rat Fas ligand (FasL) from a CTL (cytotoxic T lymphocyte) cell line (Suda and Nagata, 1994). The purified FasL had an M_r of 40 kd, and the isolation of its cDNA indicated that FasL is a member of the TNF family (Suda et al., 1993). In this report, we have cloned and localized the mouse *Fasl* gene to the *gld* region of mouse chromosome 1 by interspecific backcross analysis. Splenocytes of wild-type and *gld* mice express *Fasl* mRNA upon activation. However, the protein coded by *gld* mice carries a point mutation and cannot

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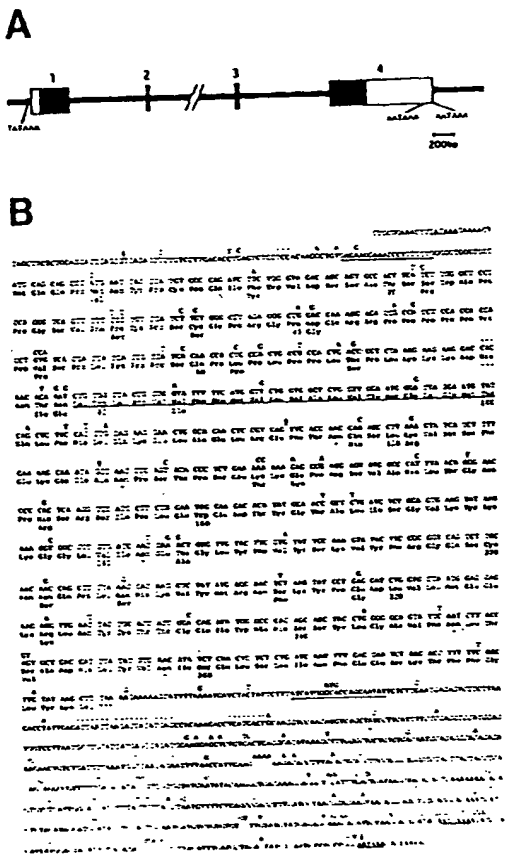

Figure 1. The Structure of Mouse *Fasl*

(A) The gene organization of mouse *Fasl*. The organization of the mouse *Fasl* gene is shown schematically. Boxes and the lines between them represent four exons and three introns, respectively. The coding sequence is represented by the filled area, while the open area indicates the noncoding sequence.

(B) Nucleotide sequence and predicted amino acid sequence of mouse *Fasl*. The nucleotide sequence and the predicted amino acid sequence of exons and the promoter region of the mouse *Fasl* gene are shown with the corresponding rat sequences (Suda et al., 1993). The rat nucleotide and amino acid sequences that are different from those in mouse *Fasl* are indicated above the nucleotide sequence and below the amino acid sequence of mouse *Fasl*, respectively. Arrowheads indicate the positions of introns. Two vertical arrows indicate the start and end points of the rat *Fasl* cDNA. Comparison of mouse and rat sequences was done within this region. The numbers below each line indicate the amino acid position of mouse FasL. The TATAAA box, polyadenylation signal (AATAAA), and the putative transmembrane region are underlined, while five potential N-linked glycosylation signals (Asn–X–Ser/Thr) are indicated by asterisks. The primers used for reverse PCR are indicated by horizontal arrows under the nucleotide sequence.

induce apoptosis in cells expressing Fas. These results indicate that the abnormal phenotypes such as lymphoproliferation and autoimmune disease in *gld* mice are due to a mutation in *Fasl*.

Results

Isolation of the Mouse Fas Ligand Gene

Screening of a mouse genomic library prepared from mouse 129/Sv strain with the rat *Fasl* cDNA yielded two positive clones (λMFL5 and λMFL18). Restriction mapping and Southern blot hybridization analysis of these clones indicated that λMFL5 and λMFL18 carry the 5′ and 3′ part of the *Fasl* gene, respectively. The nucleotide sequence of the genomic regions corresponding to the rat *Fasl* cDNA, in addition to part of the promoter region, was determined. The sequence revealed a high conservation with the rat *Fasl* sequence, suggesting that cloned λDNAs carry the mouse *Fasl* gene. Comparison of the nucleotide sequence of the mouse *Fasl* gene with that of the rat *Fasl* cDNA (Suda et al., 1993) revealed the genomic organization of mouse *Fasl*. As shown in Figure 1A, the mouse *Fasl* gene consists of four exons. All the splice donor and acceptor sites conform to the GT——AG rule (Padgett et al., 1986) for nucleotides immediately flanking exon borders. Further flanking sequences are in good agreement with favored nucleotide frequencies noticed in other split genes (Padgett et al., 1986). The gene organization of mouse *Fasl* is similar to that of other members of the TNF family such as TNFα and lymphotoxin β (Browning et al., 1993; Nedwin et al., 1985).

Figure 1B shows the nucleotide sequence of the promoter, exons, and 3′ flanking region of mouse *Fasl* gene, together with the corresponding sequence of rat *Fasl* cDNA. There is a long open reading frame of 837 bp starting from the ATG initiation codon located 107 bp downstream of the TATA box. The open reading frame codes for a protein of 279 amino acids with a calculated M_r of 31,440. The nucleotide and amino acid sequences of mouse *Fasl* have identities of 90.6% and 91.4% with rat *Fasl*, respectively. Even the 3′ noncoding region of the mouse *Fasl* gene has an identity of 84.5% with that of rat *Fasl*. Similar to rat FasL (Suda et al., 1993), the mouse FasL contains no signal sequence at the N terminus, but contains a stretch of hydrophobic amino acids (22 residues) in the middle of the molecule, suggesting that mouse FasL is a type II membrane protein. The cytoplasmic region consisting of 78 amino acids is rich in proline residues; 25 out of 78 residues are proline. The C-terminal extracellular region consists of 179 amino acids, contains five potential N-glycosylation sites (Asn–X–Ser/Thr), and has a significant similarity with other members of the TNF family.

Murine Chromosomal Location of the Fas Ligand

The murine chromosomal location of *Fasl* was determined by interspecific backcross analysis using progeny derived from matings of (C57BL/6J × Mus spretus)F1 × C57BL/6J mice. This interspecific backcross mapping panel has been typed for over 1500 loci that are well distributed among all the autosomes as well as the X chromosome (Copeland and Jenkins, 1991). C57BL/6J and M. spretus DNAs were digested with several restriction enzymes and analyzed by Southern blot hybridization for informative

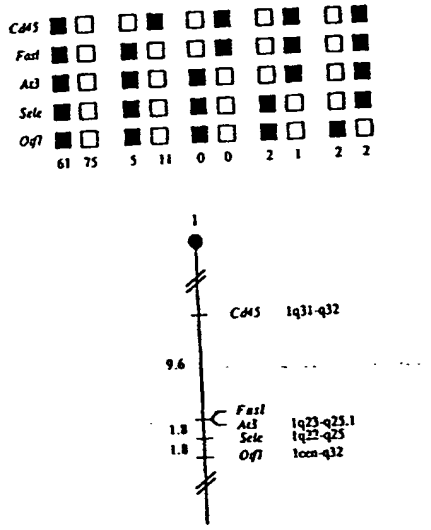

Figure 2. *Fasl* Maps in the Distal Region of Mouse Chromosome 1

Fasl was placed on mouse chromosome 1 by interspecific backcross analysis. The segregation of *Fasl* and flanking genes in 158 backcross animals that were typed for all loci is shown at the top of the figure. For individual pairs of loci, more than 158 animals were typed (see text). The shaded boxes represent the presence of a C57BL/6J allele, and white boxes represent the presence of an M. spretus allele. The number of offspring inheriting each type of chromosome is listed at the bottom of each column. A partial chromosome 1 linkage map showing the location of *Fasl* in relation to linked genes is shown at the bottom of the figure. Recombination distances in centimorgans between loci are shown to the left of the chromosome, and the positions of loci in human chromosomes, where known, are shown to the right. References for the human map positions of loci mapped in this study can be obtained from GDB (Genome Data Base), a computerized database of human linkage information maintained by the William H. Welch Medical Library of the Johns Hopkins University (Baltimore, Maryland).

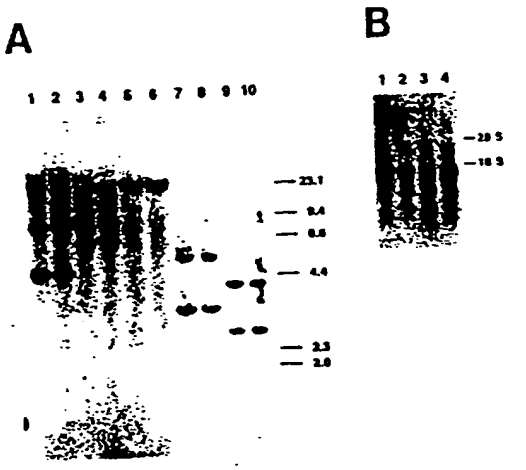

Figure 3. Southern and Northern Hybridization Analysis of the *Fasl* Gene

(A) No gross rearrangement of the *Fasl* gene in *gld* mice. Genomic DNA (10 μg per lane) from C3H/HeJ(+/+) (lanes 1, 3, 5, 7, and 9) and C3H/HeJ(*gld*/*gld*) (lanes 2, 4, 6, 8, and 10) was digested with BamHI (lanes 1 and 2), EcoRI (lanes 3 and 4), HindIII (lanes 5 and 6), PstI (lanes 7 and 8), or XbaI (lanes 9 and 10). The DNA was electrophoresed in a 0.8% agarose gel and transferred to a nitrocellulose filter. Hybridization was carried out under high stringency with ^{32}P-labeled mouse *Fasl* cDNA carrying the entire coding sequence. HindIII-digested λDNA was electrophoresed in parallel, and the sizes of the fragments are indicated to the right in kilobases.

(B) Expression of *Fasl* mRNA in activated splenocytes. Splenocytes were prepared from C3H/HeJ(+/+) (lane 1), C3H/HeJ(*gld*/*gld*) (lane 2), MRL/MpJ(+/+) (lane 3), and MRL/MpJ(*lpr*/*lpr*) (lane 4). The splenocytes were activated with PMA and ionomycin as described under Experimental Procedures, and poly(A) RNA was prepared. The RNA (2 μg per lane) was then analyzed by Northern blot hybridization using ^{32}P-labeled mouse *Fasl* cDNA as probe.

restriction fragment length polymorphisms (RFLPs), using a probe from exon 4 of the mouse *Fasl* genomic locus. A 1.9 kb M. spretus SphI RFLP (see Experimental Procedures) was used to follow the segregation of the *Fasl* locus in backcross mice. As shown in Figure 2, the mapping results indicated that *Fasl* is located in the distal region of mouse chromosome 1 linked to *Cd45*, *At3*, *Sele*, and *Otf1*. No recombination was detected between *Fasl* and *At3* in 180 animals typed in common, suggesting that the two loci are within 1.7 cM of each other (upper 95% confidence limit). Previous mapping experiments have placed *gld* within 0.56 ± 0.39 cM of *At3* on chromosome 1 (Watson et al., 1992). Our mapping of *Fasl* within 1.7 cM of *At3* places *Fasl* in the same chromosomal location as *gld*, consistent with the hypothesis that *Fasl* encodes *gld*.

A Missense Point Mutation in the Fas Ligand from *gld* Mice

To examine whether *gld* mice carry a mutation in the *Fasl* gene, chromosomal DNAs were prepared from spleens of the wild-type and *gld* mice, digested with BamHI, EcoRI, HindIII, PstI, or XbaI, and subjected to Southern blot hybridization analysis. The 0.9 kb DNA fragment carrying the complete coding sequence (see below) was used as a probe. As shown in Figure 3A, the wild-type mouse DNA digested with various restriction enzymes gave a few bands. The sizes of the hybridizing bands were consistent with the restriction map of the cloned genomic locus of mouse *Fasl*, indicating that there is only one genomic gene for *Fasl* in the mouse haploid genome. When genomic DNA from *gld* mice was analyzed by Southern blot hybridization, the bands were indistinguishable from those seen in wild-type DNA for all restriction enzymes analyzed (Figure 3A). These results indicate that no gross rearrangements have occurred in the *Fasl* gene of *gld* mice.

Activation of rat splenocytes with phorbol-12-myristate-13-acetate (PMA) and ionomycin induces expression of *Fasl* mRNA (Suda et al., 1993). To examine the expression of *Fasl* in *gld* mice, splenocytes were prepared from wild-type (C3H[+/+], MRL[+/+]) and mutant (C3H[*gld*/*gld*],

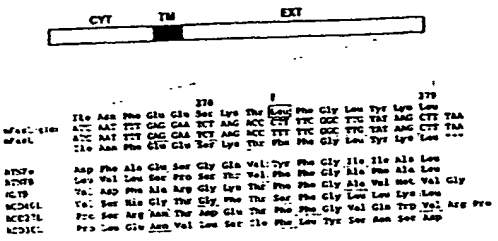

Figure 4. Point Mutation in the FasL of *gld* Mice

The upper panel shows the predicted structure of mouse FasL. CYT, TM, and EXT indicate the cytoplasmic region, transmembrane domain, and the extracellular region, respectively. The lower panel shows the nucleotide sequence and the deduced amino acid sequence of the *Fasl* cDNA of *gld* (mFasL–*gld*) and wild-type (mFasL) mice at the site of the mutation. Numbers above the line indicate the amino acid positions of mouse FasL. The arrowhead indicates the position of the mutation in the *Fasl* gene of *gld* mice. The mutated amino acid (Leu-273) is boxed by a solid line. Amino acid sequences of the corresponding region of human TNFα (hTNFα), human TNFβ (hTNFβ), human lymphotoxin β (hLTβ), human CD40 ligand (hCD40L), human CD27 ligand (hCD27L), and human CD30 ligand (hCD30L) are included. The amino acids of favored substitutions in more than four members are boxed by a dotted line.

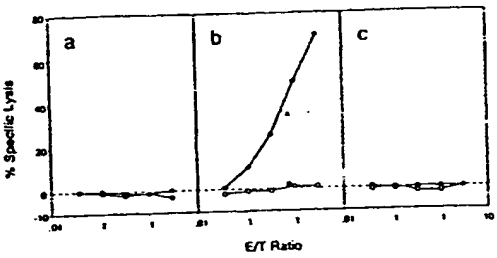

Figure 5. Inability of *gld* FasL to Induce Apoptosis

COS cells were transfected with pEF-BOS vector (a) or pEF-BOS carrying the wild-type *mFasl* cDNA (b) or the *gld* *mFasl* cDNA (c). At 48 hr after transfection, cytotoxicity of the transfected COS cells was assayed using W4 (closed circles) or WR19L cells (open circles) as target cells. The effects of the soluble form of Fas or TNF receptor on the cytotoxic activity of the wild-type recombinant mFasL (b) was examined by adding 20 μg/ml of mFas–Fc (closed square) or hTNFRβ–Fc (closed triangle) to the assay mixture at an E/T (effector cells/target cells) ratio of 0.75, as described previously (Suda et al., 1993).

MRL[*lpr*/*lpr*]) mice. After activation with PMA and ionomycin, poly(A) RNAs were prepared from the splenocytes and analyzed by Northern blot hybridization. As shown in Figure 3B, poly(A) RNAs from all mouse strains gave a band of about 2 kb hybridizing with mouse *Fasl* probe DNA. The detection of *Fasl* mRNA of apparently intact size in *gld* mice indicates that the mice can produce *Fasl* mRNA as efficiently as the wild-type mice.

To determine the coding sequence of the mouse *Fasl* of *gld* mice, a set of oligonucleotide primers representative of the 5′ or 3′ noncoding region (see Figure 1B) were prepared. Using these primers, the coding sequence of mouse *Fasl* was amplified by polymerase chain reaction (PCR) after reverse transcription of mRNA from the activated splenocytes of wild-type or *gld* mice. The resultant 0.9 kb PCR product was inserted into pBluescript II, and its nucleotide sequence was determined. The PCR product from wild-type C3H mice had a sequence identical to that found in 129/Sv mice (see Figure 1B). On the other hand, six independent *Fasl* cDNA clones of *gld* mice, derived from two independent PCR reactions, showed a transition of T to C near the 3′ end of the coding sequence (Figure 4). This mutation causes the replacement of phenylalanine with leucine at the amino acid position 273 in the extracellular region of the mouse FasL, which is highly conserved among members of the TNF family.

To establish whether this mutation abolishes the ability of FasL to induce apoptosis in cells expressing Fas, *Fasl* cDNAs from wild-type and *gld* mice were expressed in COS cells. The cytotoxic activity of COS cells expressing recombinant FasL was then examined using WR19L transformants (W4) that express mouse Fas (Ogasawara et al., 1993). As shown in Figure 5, the COS cells transfected with wild-type mouse *Fasl* cDNA could kill the W4 cells but not the WR19L parental cells in a dose-dependent manner. Furthermore, the cytotoxic activity of COS cells transfected with mouse *Fasl* cDNA could be inhibited by a soluble form of human TNF receptor (TNFR–Fc). These results confirm that the *Fasl* cDNA cloned from wild-type mice is functional and can induce apoptosis by binding to Fas. However, the COS cells transfected with *gld* *Fasl* cDNA showed little cytotoxic activity. These results indicate that the FasL expressed in *gld* mutant mice is unable to induce apoptosis in Fas-expressing cells, and that *gld* is a mutation of the *Fasl*.

Discussion

Mice carrying either *lpr* or *gld* mutation suffer from lymphadenopathy and autoimmune disease (Cohen and Eisenberg, 1991). Previously, we showed that the *lpr* mutation is a mutation of the *Fas* gene, which encodes for a cell-surface protein mediating apoptosis (Adachi et al., 1993; Watanabe-Fukunaga et al., 1992a). In this report, we have shown that the *gld* mice carry a defect in FasL. These results support the hypothesis, predicted by bone marrow transplantation (Allen et al., 1990), that *gld* and *lpr* are mutations of genes encoding a cytokine and its receptor, respectively.

FasL from *gld* mice contains a point mutation in the C-terminal region of the molecule, a region which is highly conserved among members of the TNF family. According to the structural model of human TNFα (Eck and Sprang, 1989; Jones et al., 1989), this region forms a β sheet and is situated inside the molecule. Several groups have generated mutants of human TNFα (Gase et al., 1990; Van Ostade et al., 1991; Yamagishi et al., 1990). In agreement

with our results for *gld* FasL, mutations in the corresponding C-terminal region of the TNFα severely affect the ability of TNFα to bind the receptor (Gase et al., 1990). Since it is unlikely that this region is directly involved in receptor binding, the *gld* mutation may induce distortion of the molecule affecting the receptor binding site or disturb the formation of the biologically active trimer (Smith and Baglioni, 1987). Furthermore, since C-terminal mutations in TNFα still retain some activity (Gase et al., 1990; Yamagishi et al., 1990), our results for *gld* do not rule out the possibility that the mutation is leaky.

As described above, *lpr* and *gld* mutations behave as loss-of-function mutations. The lymphadenopathy and autoimmune disease observed in these mice therefore suggest that the Fas system plays an important role in the apoptotic process that takes place during development of T cells. Since neonatal thymectomy prevents lymphoid organ hyperplasia and early autoimmune disease in *lpr* mice (Hang et al., 1984), these mice have been thought to have defects in the intrathymic development of T cells. Consistent with this hypothesis, most mouse thymocytes express Fas (Drappa et al., 1993; Ogasawara et al., 1993), and $CD4^+$ $CD8^+$ double-positive thymocytes are susceptible to the cytolytic activity of anti-Fas antibody or Fas ligand (J. Ogasawara, T. S., and S. N., unpublished data). However, it remains unclear whether the Fas system is involved in the thymic development of T cells. Several groups have reported that T cell deletion mediated by endogenous superantigens (clonal deletion) is essentially normal in *lpr* mice (Giese and Davidson, 1992; Herron et al., 1993; Kotzin et al., 1988), although others noticed some increase of autoreactive T cells ($V\beta8^+$ or $V\beta6^+$ cells) in these mice (Matsumoto et al., 1991; Mountz et al., 1986). The role of the Fas system in positive selection is also controversial. Zhou et al. (1993) reported that the neglected thymocytes escape from apoptosis in the thymus of *lpr* mice, then migrate to the periphery. Examination of the T cell receptor repertoire in *lpr* mice (Herron et al., 1993), and *lpr* or *gld* mice carrying the T cell receptor transgene, also has indicated that positive selection as well as negative selection is essentially normal in these mice (Sidman et al., 1992). We have not been able to detect the *Fasl* mRNA in the thymus, except for a weak signal in activated thymocytes (Suda et al., 1993), again suggesting that the Fas system may not be involved in thymic development of most T cells. However, it will be necessary to examine, by in situ hybridization, immunohistochemistry, or both, whether some limited yet specific cell populations in the thymus express FasL.

In addition to the thymus, mature T cells reacting with the self-component are deleted in the periphery (Jones et al., 1990; Kabelitz et al., 1993; Kawabe and Ochi, 1991; Russell et al., 1991). Fas is expressed in activated mature T cells (Trauth et al., 1989), and prolonged activation of mature T cells induces in the cells susceptibility to the cytolytic activity of the anti-Fas antibody (Klas et al., 1993; Owen-Schaub et al., 1992). Recent studies on mature T cells from *lpr* and *gld* mice (Russell et al., 1993; Russell and Wang, 1993) indicated that these T cells are resistant to anti-CD3-stimulated suicide. Furthermore, mature T cells from *lpr* mice showed some defect in deleting of the $V\beta8^+$ T cells following in vivo administration of superantigen (Scott et al., 1993). These results suggest a role of Fas-mediated apoptosis in the induction of peripheral tolerance. In accordance with this model, Fas was detected on 30%–50% of both activated CD4 and CD8 single-positive cells (Drappa et al., 1993), and activation of mature T cells induces the expression of *Fasl* (Figure 3B; Suda et al., 1993). It is possible that the activated T cells expressing both Fas and FasL are killed by themselves or by interacting with each other. Such a mechanism may operate not only to delete the autoreactive T cells but also to remove the activated T cells for foreign antigens after they have done their job. Furthermore, the expression of Fas in activated B cells (Drappa et al., 1993; Trauth et al., 1989), as well as the involvement of B cells in the *lpr* phenotype (Sobel et al., 1991), suggests that the T cells expressing FasL may play a role in deleting activated B cells.

Since some cytotoxic T cell lines such as PC60-d10S express FasL (Rouvier et al., 1993; Suda et al., 1993), and CTL in peritoneal exudate lymphocytes shows Fas-dependent cytotoxic activity (Rouvier et al., 1993), we postulated previously that CTL, at least in part, uses the Fas system to kill target tumor cells (Nagata, 1994; Suda et al., 1993). Recent findings by Vignaux and Golstein (1994) that mixed lymphocyte cultures from *gld* mice do not show Fas-dependent cytotoxic activity agree with our results that *gld* mice carry a nonfunctional mutation in FasL, and they confirm that the Fas system plays a role in T cell–mediated cytotoxicity. Various human diseases such as Graves' disease, chronic thyroiditis, and fulminant hepatitis are suggested to be mediated by cytotoxic T cells (Rose and Bona, 1993). It would be interesting to examine whether FasL plays a role in the pathogenesis of these diseases.

Experimental Procedures

Isolation of the Genomic Clones for Mouse Fas Ligand

Plaques (1.3×10^6) from a mouse genomic library (Stratagene, La Jolla, California) constructed with DNA from 129/Sv mouse and Lambda FIX II vector were screened by plaque hybridization. A 568 bp DNA fragment from nucleotides 400 to 967 of pTN24-15 or a 190 bp DNA fragment from 43 to 233 of pTN24-15 was used as 3′ or 5′ probe DNA, respectively. The probe DNAs were prepared by PCR and labeled with ^{32}P, using a random primer labeling kit (Boehringer Mannheim). Hybridization was carried out at low stringency. In brief, after hybridization at 33°C for 18 hr, the filters were washed twice at room temperature with 2 × SSCP (1 × SSCP is 150 mM NaCl, 15 mM Na_3 citrate, 10 mM NaH_2PO_4, 1 mM EDTA [pH 7.2]) containing 0.1% SDS, and three times at 37°C with 0.3 × SSCP containing 0.1% SDS. Positive clones were plaque purified, and the inserted mouse DNAs were subjected to restriction enzyme mapping and DNA sequencing analysis after subcloning into pBluescript II (Stratagene).

Interspecific Backcross Mapping

Interspecific backcross progeny were generated by mating (C57BL/6J × M. spretus) F1 females and C57BL/6J males as described (Copeland and Jenkins, 1991). A total of 205 N2 mice were used to map the *Fasl* locus (see text for details). DNA isolation and Southern blot hybridization were performed essentially as described (Jenkins et al.,

1982). All blots were prepared with Hybond N⁺ membrane (Amersham). The probe, a 1.4 kb SpeI fragment of mouse genomic DNA carrying exon 4 of the *Fasl* genomic locus, was labeled with [α-^{32}P]dCTP using a nick translation kit (Amersham); washing was done to a final stringency of 0.1 × SSCP, 0.1% SDS, 65°C. A fragment of 11.8 kb was detected in SphI-digested C57BL/6J DNA, and a fragment of 1.9 kb was detected in SphI-digested M. spretus DNA. The presence or absence of the 1.9 kb M. spretus–specific SphI fragment was followed in backcross mice.

A description of the probes and RFLPs for one locus linked to *Fasl* including CD45 antigen (*Cd45*, formerly *Ly5*), antithrombin 3 (*At3*), selectin endothelium (*Sele*, formerly *Elam*), and octamer-binding factor 1 (*Otf1*) has been reported previously (Singh et al., 1991; Siracusa et al., 1991). Recombination distances were calculated as described (Green, 1981) using the computer program SPRETUS MADNESS. Gene order was determined by minimizing the number of recombination events required to explain the allele distribution patterns.

Cloning of Mouse Fas Ligand cDNA by PCR

Splenocytes from wild-type (C3H[*+/+*]) or *gld* (C3H[*gld/gld*]) mice were incubated at a concentration of 2 × 10⁶ cells/ml in RPMI 1640 medium (Nissui, Tokyo) containing 10% fetal calf serum (FCS), 50 μM β-mercaptoethanol, 1.5 μg/ml Con A and 20 ng/ml interleukin-2 at 37°C for 2 days. The Con A blasts were then treated with 10 ng/ml PMA and 500 ng/ml ionomycin for 4 hr. After treatment, dead cells were removed by density gradient centrifugation using Histopeque 1083 (Sigma), and poly(A) RNA was prepared using an mRNA isolation kit from Pharmacia. Single-stranded cDNA synthesis and PCR were carried out as described by Kawasaki (Kawasaki, 1990). In brief, 1 μg of poly(A) RNA was used as a template for cDNA synthesis in 20 μl of reaction mixture with 50 ng of random hexamer and 200 U of M-MLV RNAase H⁻ reverse transcriptase (GIBCO BRL). An aliquot (1.0 μl) of the reaction mixture was diluted with 100 μl of PCR buffer containing 100 pmol each of the sense and antisense primers. The sense primer carries the 20 nt sequence (GAGAAGGAAACCCTTTCCTG) upstream of the ATG initiation codon and an XbaI recognition site (GCTCTAGA) at the 5′ end, whereas the downstream primer carries the sequence (ATATTCCTGGTGCCCATGAT) downstream of the TAA termination codon and an XbaI site. The reaction mixture was placed in a DNA thermal cycler (Perkin-Elmer Cetus), and the reaction was started by adding 2.5 U of Thermus aquaticus DNA polymerase (Taq polymerase; Takara Shuzo Company, Kyoto). The conditions for the PCR were 1.0 min at 94°C, 2 min at 55°C, and 3 min at 72°C for 20 cycles. The PCR products were digested with XbaI and fractionated on 1% agarose gel (Low Gel Temperature, Bio-Rad). A 940 bp DNA fragment was recovered from the gel and subcloned into the XbaI site of pBluescript II.

Transfection of COS Cells and Assay for Cytotoxic Activity

The 940 bp XbaI DNA fragment carrying mouse *Fasl* cDNA was inserted into the XbaI site of pEF-BOS, a mammalian expression vector (Mizushima and Nagata, 1990). Monkey COS cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Nissui Company, Tokyo) containing 10% FCS. COS cells (2 × 10⁶) on 10 cm plates were transfected with 5 μg of plasmid DNA by the DEAE–dextran method (Fukunaga et al., 1990), and the cytotoxic activities of the transfected cells were determined as described previously (Suda et al., 1993). In brief, 1 × 10⁶ WR19L or W4 cells (Ogasawara et al., 1993) were labeled with ^{51}Cr by incubation at 37°C for 2 hr in RPMI 1640 medium containing 20 μCi of [^{51}Cr]sodium chromate (Amersham). These ^{51}Cr-labeled cells (1 × 10⁴) were mixed with transfected COS cells at various ratios, and the release of ^{51}Cr was determined after incubation for 4 hr at 37°C.

General Procedures

DNA sequencing was carried out using a DNA sequencer (model 370A, Applied Biosystems) and a Taq DyeDeoxy Terminator cycle sequencing kit from Applied Biosystems. In some cases, synthetic oligonucleotides were used as specific primers.

Northern and Southern blot hybridizations were carried out under high stringency conditions with a ^{32}P-labeled 940 bp DNA fragment carrying mouse *Fasl* cDNA as a probe.

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